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Transmembrane mobility and distribution of phospholipids in the membrane of mouse β-thalassaemic red blood cells

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Using spin-labelled lipid analogues, the transmembrane mobility and distribution of phospholipids in normal and β-thalassaemic murine red blood cells were investigated. The velocities of spin-labelled phosphatidylserine (PS*) and spin-labelled phosphatidylethanolamine (PE*) active transport into the inner leaflet were not significantly different between normal and pathological cells. The stationary distribution of PE* in thalassaemic erythrocytes (79.5 ± 2.0% inside) differed from that of control cells (91.1 ± 1.6% inside), while that of PS* was unaffected. In thalassaemic cells the passive diffusion of spin-labelled phosphatidylcholine (PC*) was accelerated 4-fold and its stationary distribution was shifted to 34.5 ± 2.3% inside compared to 19.5 ± 1.6% in control cells. Spin-labelled sphingomyelin (SM*), which showed no inward movement in normal cells, diffused partially towards the inner leaflet of thalassaemic erythrocyte membranes. These results indicate that modifications of the transverse lipid organisation in β-thalassaemic red blood cells are due to changes in passive diffusion movements, and not to changes in aminophospholipid translocase activity.

Introduction

The β-thalassaemia syndromes are well-characterised in primary molecular defects leading to the decreased expression or absence of the β-globin chain and the subsequent accumulation of unpaired α-haemoglobin chains which are unstable and associate with the membrane [1]. In contrast, the mechanisms leading to the premature destruction of β-thalassaemic erythroblasts in the bone marrow and of red blood cells in circulation are poorly understood. A number of biochemical alterations of thalassaemic erythrocytes have been described (reviewed in Ref. 2) including changes in membrane cholesterol, phospholipids and fatty acids [3] and various protein defects [4–6], suggestive of an increased oxidation level associated with abnormal amounts of heme derivatives [7] and non-heme iron [8] in the membrane.

To progress in the understanding of the pathophysiology of the anaemia in β-thalassaemia, the mouse model of the pathology offers the advantage of the lack of heterogeneity seen in the human disease and of membrane defects which are similar to that observed in human β-thalassaemic red cells, for the same degree of anaemia [9–10]. For these reasons we have investigated the transmembrane mobility and distribution of phospholipids in murine β-thalassaemic red cells.

The asymmetric transverse distribution of phospholipids between both leaflets of plasma membrane of normal red blood cells is well-established, i.e., the aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) are mainly localised in the cytoplasmic membrane leaflet, whereas the lipids with a choline headgroup, phosphatidylcholine (PC) and sphingomyelin (SM), are predominantly present in the outer monolayer [11]. The asymmetry of PS and PE is controlled by aminophospholipid translocase, which actively transports PS and PE from the outer towards the inner membrane leaflet, whereas PC and SM traverse the membrane by a passive diffusion process [12,13]. In normal red blood cells it was suggested that a change of the transverse lipid asymmetry of senescent cells,

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Abbreviations: PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; PS*, spin-labelled phosphatidylserine; PE*, spin-labelled phosphatidylethanolamine; PC*, spin-labelled phosphatidylcholine; SM*, spin-labelled sphingomyelin; HBS, Hepes-buffered saline; BSA, bovine serum albumin; EPR, electron paramagnetic resonance.

namely appearance of PS in the outer leaflet, may serve as a signal for the cell elimination from the blood stream [14]. In the case of the pathological sickled cell, it was also shown that a modification of the aminophospholipid translocase activity resulting in a perturbed transverse lipid distribution was associated with their premature removal from the circulation [15,16].

In the present study, the transverse distribution and mobility was measured by using spin-labelled lipid analogues of the four main endogenous phospholipids [17]. The spin-labels having a short (five carbons) *sn*-2 chain which bears the nitroxide radical can be easily incorporated into the outer membrane leaflet. At any time, the amount of label in the outer monolayer can be estimated by back-exchange onto bovine serum albumin.

Materials and Methods

Preparation of erythrocytes. Blood was drawn from DBA/2J control and thalassaemic mice (Hbb th1/th1) and used on the same day after three washes in Hepes-buffered saline (HBS; 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.1 mM EGTA, 10 mM glucose, 10 mM inosine, 20 mM Hepes (pH 7.4)).

Spin labelling and EPR determination of the transmembrane distribution of spin-labelled lipids. 1-palmitoyl-2-(4-doxyl-pentanoyl)-phosphatidylcholine (PC*), -phosphatidylserine (PS*), -phosphatidylethanolamine (PE*) and *N*-(4-doxylpentanoyl)-*trans*-sphingenyl-1-phosphocholine (SM*) were synthesised as described [12,17]. An aliquot, corresponding to 1% of the endogenous phospholipids in the final incubation, of the desired analogue in chloroform solution was deposited in a glass tube, dried under vacuum and resuspended by vigorous vortexing with HBS buffer. Blood sample and label suspension were prewarmed at 37°C and the translocation assay was initiated by mixing 1 vol. of phospholipid suspension to 2 vols. of erythrocyte suspension at 50% hematocrit. To minimise the hydrolysis of spin-labelled phospholipids, di-isopropylfluorophosphate (5 mM) was added to all samples. The determination of the lipid transmembrane distribution was performed by the back-exchange technique [17]. Briefly, 120-μl aliquots were taken from the labelled erythrocytes suspension at given times, mixed with 30 μl of 4% fatty acid-free BSA and incubated on ice for 1 min. After centrifugation (30 s, $7600 \times g$ in an Eppendorf tube), the supernatant was taken and analysed by electron paramagnetic resonance (EPR) spectroscopy after addition of 10 mM potassium hexaferricyanide to reoxidize all of the label. Comparison of the signal associated to the pellets of aliquots treated at time zero with and without BSA demonstrated that more than 97% of the analogue was recovered in the BSA-containing supernatant. Data were fitted with exponential equations using the KaleidaGraph data analysis and graphics application (Abelbeck Software).

Recording of the membrane spectra. The spin-labelled lipids were introduced into the erythrocyte membranes as for the kinetics assay. After a 10-min incubation at 37°C, cells were pelleted by centrifugation and mixed with potassium hexaferricyanide (final concentration 10 mM). All EPR measurements were performed with a Varian E-109 spectrometer equipped with a temperature control device.

Miscellaneous. Hemolysis was estimated by measuring photometrically, at a wavelength of 540 nm, the haemoglobin content in the incubation supernatants. Statistical comparisons were done by using the *t*-test (paired observation, $\alpha = 0.05$).

Results

*Transmembrane reorientation of PS**

The inward motion of PS* in the membranes of control and β-thalassaemic mouse red blood cells at 37°C or 20°C was too fast to allow several aliquots to be taken out before the steady-state transmembrane distribution was reached. Therefore, the inward motion was measured at 4°C, a temperature at which the kinetics could be analysed (Fig. 1). At any temperature, steady-state distribution of the PS analogue was not significantly different in control cells and in β-thalassaemic cells. On the other hand, the initial velocity of reorientation was slightly higher in healthy cells ($3.72 \pm 0.49\%\,min^{-1}$) than in pathological ones ($3.09 \pm 0.22\%\,min^{-1}$).

*Transmembrane reorientation of PE**

The transverse asymmetry of PE* was significantly reduced in thalassaemic cells compared to control cells

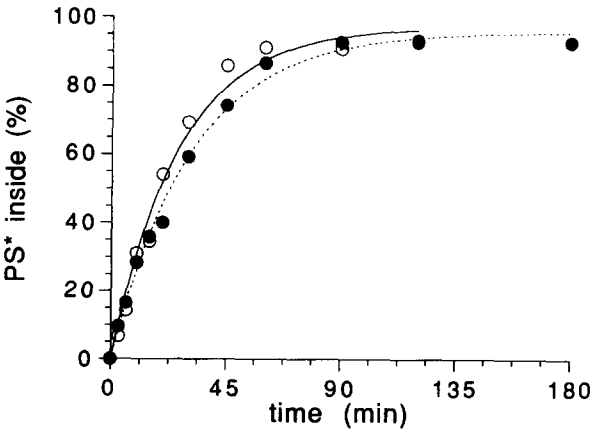

Fig. 1. PS* transport kinetics into the inner membrane leaflet. Experiments were carried out at 4°C, as described in Materials and Methods, with healthy (○) or β-thalassaemic (●) red cells. Data shown here and in other figures, correspond to a representative experiment. Curves are fitted as described in Materials and Methods.

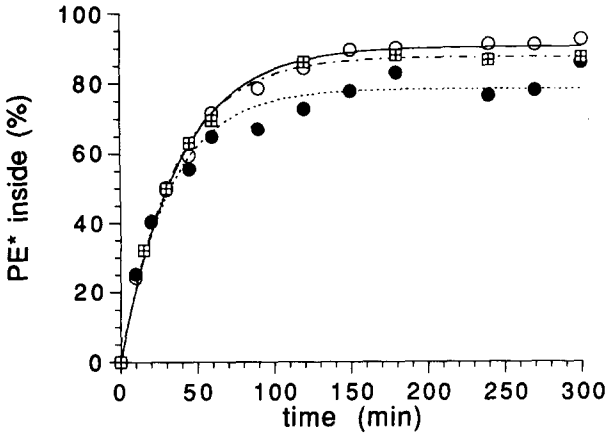

Fig. 2. PE* transport kinetics into the inner membrane leaflet. Experiments were carried out at 37°C, as described in Materials and Methods, with blood from healthy (○), β-thalassaemic (●) and bled, healthy (⊞) animals.

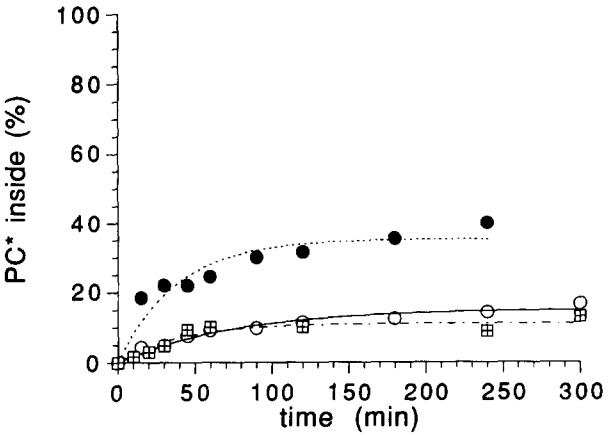

Fig. 3. PC* diffusion kinetics into the inner membrane leaflet. Experiments were carried out at 37°C, as described in Materials and Methods, with blood from healthy (○), β-thalassaemic (●) and bled, healthy (⊞) animals.

(Fig. 2 and Table I). However, no significant differences in the initial velocities of transmembrane motion could be established (Table I).

Transmembrane reorientation of PC and SM**

These lipid analogues showed much more striking differences between normal and pathological erythrocytes: PC* diffused 4-times faster and accumulated 2-times more in the inner membrane leaflet of thalassaemic cells than in the inner membrane leaflet of normal cells (Fig. 3 and Table I). Note that because of the slow movement occurring in control cells, the plateau given in Table I was resulting from curve fitting. In all eukaryotic plasma membranes investigated so far, SM* showed no or a very weak inward diffusion. While we could not find any noticeable transmembrane movement of SM* in healthy red blood cells from control mice (Fig. 4 and Table I), SM* was able to diffuse towards the inner membrane leaflet of thalassaemic cells, reaching a steady-state of 27.1 ± 4.2% of the probe in the inner monolayer (Fig. 4 and Table I).

TABLE I

Stationary distribution and initial velocity of inward relocation of phospholipid analogues in control and β-thalassaemic cells at 37°C

Values are given ± standard error of estimate, *n* is the number of experiments.

Cell	Analogue	*n*	Plateau (%)	Initial velocity (% min^{-1})
Control	PE	5	91.1 ± 1.6	2.33 ± 0.13
	PC	6	19.5 ± 1.6	0.35 ± 0.09
Thalassaemic	PE	5	79.5 ± 2.0	2.80 ± 0.30
	PC	6	34.5 ± 2.3	1.41 ± 0.29
	SM	3	27.1 ± 4.2	0.92 ± 0.31

Lipid redistribution in red blood cells of bled mice

Blood of β-thalassaemic mice is characterised by an increased proportion of reticulocytes. Therefore, we measured the transmembrane reorientation in blood cells from healthy mice where reticulocyte production was stimulated by drawing 0.2 ml blood daily for three successive days. Lipid reorientation kinetics were measured on the fourth day. The equilibrium distribution and initial velocity of inward diffusion of PE* or PC* showed no significant differences with those obtained with unbled animals (Figs. 2 and 3). Moreover, we could not find any SM* diffusion (Fig. 4). Thus, an increased amount of reticulocytes in thalassaemic blood could not be responsible for the experimental differences.

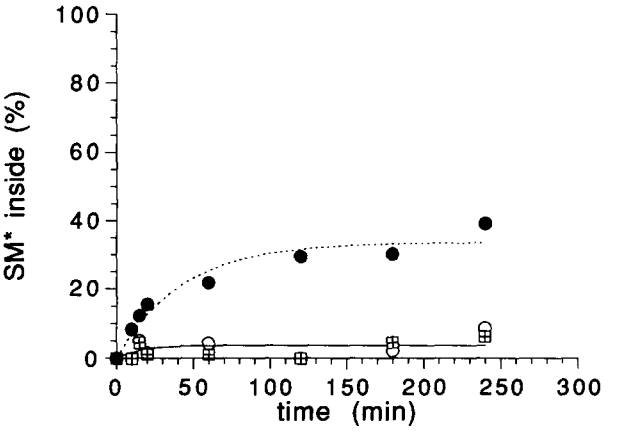

Fig. 4. SM* diffusion kinetics into the inner membrane leaflet. Experiments were carried out at 37°C, as described in Materials and Methods. Red cells originated from healthy (○), β-thalassaemic (●) or bled, healthy (⊞) animals.

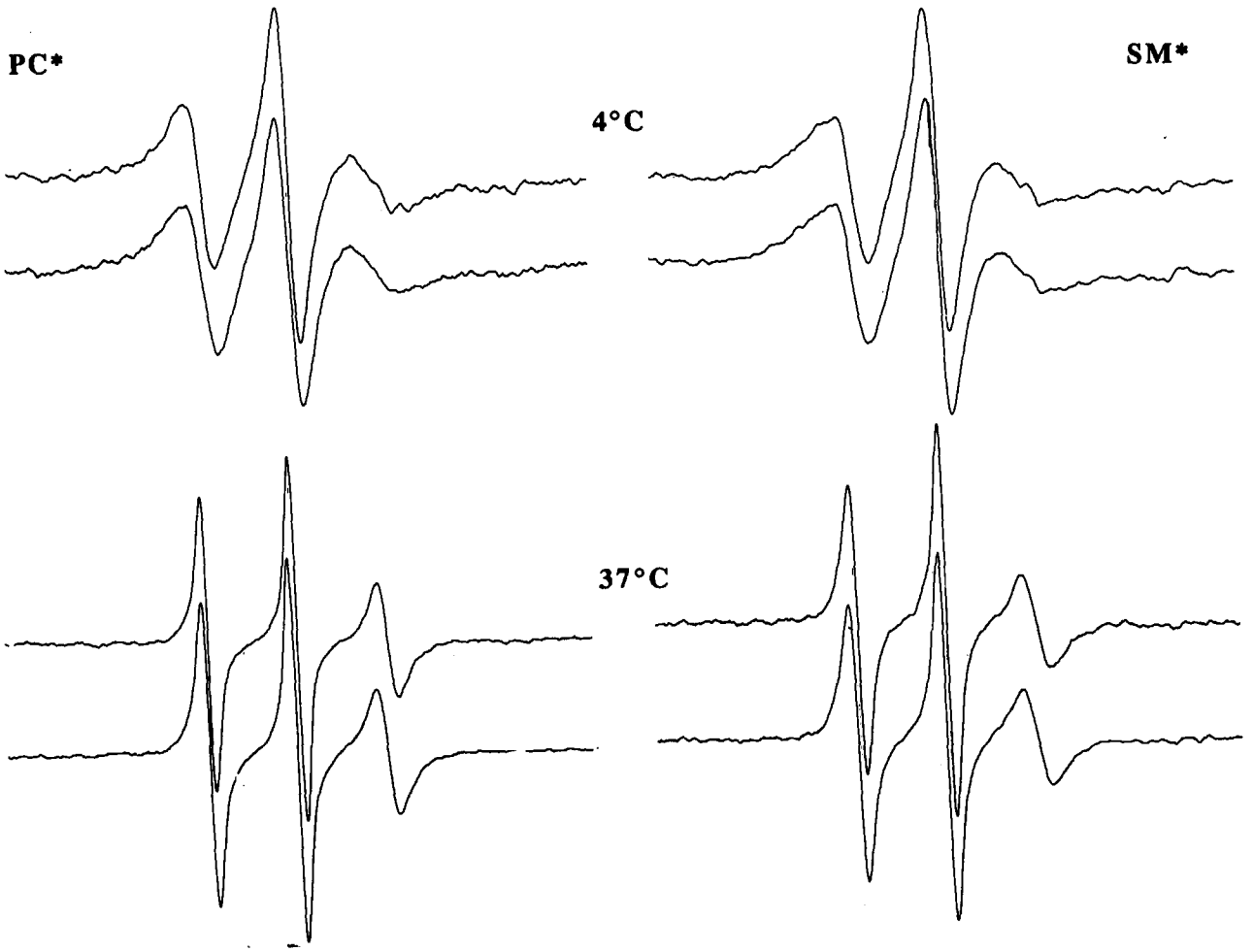

Fig. 5. EPR spectra recorded at 4°C and 37°C with either PC* or SM* embedded in the membrane of thalassaemic (top trace in each panel) or healthy (bottom trace) erythrocytes (Scan width 100 G).

EPR line shapes

Differences in the rotational mobility of spin-labelled phospholipids embedded in bilayer can be revealed by their EPR spectra, narrower lineshapes being associated with a higher mobility of the probe. These differences have been shown to be due to different composition in fatty acids of the spin-label environment [18,19]. Spectra recorded at 4°C and at 37°C with PC* and SM* exhibited a minimally narrower lineshape in thalassaemic than in control erythrocytes (Fig. 5), arguing for an almost identical outer leaflet in the two cells. In healthy and thalassaemic erythrocytes, comparison of spectra recorded from PS* equilibrated in the inner leaflet (not shown) and of PC* in the outer one were indicative of a higher mobility in the former leaflet, as in the human erythrocyte membrane [18].

Discussion

Not surprisingly, the present results are in agreement with the data obtained with erythrocytes of other mammalian species [20]. PS* and PE* crossed the membrane with a high rate constant and were preferentially localised in the inner membrane monolayer, while PC* and SM* showed a slow or no inward passage and resided mainly on the outer leaflet. The equilibrium distribution of the spin-labelled analogues in the murine erythrocyte is comparable to the transverse distribution of the endogenous phospholipids, as determined by phospholipase degradation [21].

The validity of the spin-labelled analogues to study the behaviour of the endogenous species is inferred from comparisons between reported data. In human erythrocytes, it has been shown that not only the spin-labelled analogues adopted the same localization as the endogenous molecules, but also that the presence of the paramagnetic doxyl moiety did not affect their diffusion characteristics when compared to regular diacylphospholipids [20]. The same conclusion holds when data reported here with normal murine erythrocytes are compared to a previous study in which long-chain, diacyl radioactive species were used as probes [22]. Thus, one can expect that a modification of the transmembrane movement and of the steady-state distribu-

tion of the spin-labelled analogues will reflect a similar modification in that of the endogenous molecules.

Investigations on thalassaemic cells indicated striking changes in their transverse lipid structure. The PC fraction present in the inner membrane leaflet was increased about 2-fold and the initial velocity of PC transmembrane reorientation was 4-fold more rapid in pathologic cells when compared to control cells. It has to be noted that these properties were described in an other erythrocyte type with an abnormal shape, i.e., sickled cells [15,16]. An explanation could be that the mechanical stress generated at the bilayer level by the membrane deformation increased the occurrence of 'defects' necessary for phospholipid redistribution.

In contrast to control erythrocytes and to all plasma membranes investigated up to now which showed no transverse SM movement, β-thalassaemic cells exhibited some SM inward motion. The appearance of such a movement could not be explained by an increased number of reticulocytes in the blood sample, as kinetics obtained with a sample from bled healthy mice, where the reticulocyte fraction was significantly enhanced, did not show any modification. This movement could not be due either to a drastic change in the physical properties of the thalassaemic bilayer, as the EPR spectra did not show substantial differences beetween the normal and the pathological membrane.

The net transmembrane motion and equilibrium distribution of the aminophospholipids PS and PE are the result of active transport, due to aminophospholipid translocase which is present in murine erythrocytes [22,23] and passive diffusion [13]. Parameters used to fit the experimental data with a curve give access to the rate constants for inward and outward movements [13]. Comparing healthy erythrocytes to thalassaemic cells (Table I), it appears that the main difference for PE* concerned its outward rate constant (0.0023 min^{-1} and 0.0072 min^{-1}, respectively) rather than its inward rate constant (0.0233 min^{-1} and 0.0280 min^{-1}, respectively).

At any temperature in the range 4–37°C, no striking difference was noted in PS* distribution between the two cell populations. As implied by the barely-reduced transport rate in thalassaemic cells, the pathology did not affect severely the carrier system. Its activity remained very high, and even an increase in outward movement, similar to the one experienced by PE*, could not affect the lipid transmembrane asymmetry [13].

A change of the lipid asymmetry in erythrocytes is thought to be a signal for their clearance from the blood stream. An increased level of PS in the outer membrane leaflet of erythrocytes triggers their recognition by macrophages, and it has been proposed that aged red blood cells are cleared from the circulation by this mechanism [14]. Similarly, the increased concentration of PS in the outer monolayer of the older sickled cells might be responsible for their shorter life-time [15,16]. The mechanism(s) leading to the premature clearance of β-thalassaemic red blood cells is not yet understood. Data reported here about PS distribution between the two membrane leaflets are not in favour of the involvement of this lipid in the clearance process. However, the impaired PS asymmetry was evident only in the denser normal erythrocytes [24] and sickled cells [15]. The amount of cells required in our assay prevented us from measuring phospholipid movements with density-separated murine red cells in order to detect an eventual alteration in the very denser ones. However, β-thalassaemic cells exhibited an abnormal percentage of PE in the outer membrane layer, as did the old healthy erythrocytes [24,25] and the irreversibly sickled cells [15,16]. This might be indicative of an appearance later in the cell lifetime of PS in this hemileaflet, triggering their elimination.

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